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INVESTIGATIONS OF IN VITRO CULTURE OF
HIPPEASTRUM AS A METHOD OF RAPID PROPAGATION.

THE LOUISIANA STATE UNIVERSITY AND
AGRICULTURAL AND MECHANICAL COL., PH.D., 1979

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INVESTIGATIONS OF IN VITRO CULTURE
OF HIPPEASTRUM AS A
METHOD OF RAPID PROPAGATION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
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in

The Department of Horticulture

by

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ABSTRACT

The objective of this investigation was to investigate techniques of in vitro culture which have been used with varying degrees of success in the propagation of other plants including some bulbous species. These techniques were applied to Hippeastrum in which clonal increase is often difficult and costly. The basal medium was Murashige and Skoog's (1962) cell culture medium. In brief, 17 factorial experiments investigated auxin [1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), 4-chlorophenoxypropionic acid (4-CPA) or 2,4-dichlorophenoxyacetic acid (2,4-D)] cytokinin [kinetin, 6-(α , β -dimethylallylamino) purine (2iP) or 6-benzylaminopurine (6-BA)] interactions. The effect of 2-chloroethyltrimethylammonium chloride (CCC), gibberellic acid (GA_3), coconut milk and increased concentrations of meso-inositol, thiamine·HCl, pyridoxine·HCl, nicotinic acid and glycine were investigated. The following plant tissues were used: root, leaf, ovary, peduncle, petal, anther, filament, style and young bulblets obtained from bulb cuttage.

Bulblets, 5 to 8 mm in diameter, were quartered longitudinally and cultured in vitro. Each bulblet section reorganized into a single growing point but could not be induced to proliferate further by auxin and/or cytokinin supplements to the medium. The resulting bulblets were large enough to be cut again within 12 weeks. This will allow for a 250 fold increase over the standard scale-stem fraction process.

Plantlets were obtained from 2 mm slices of ovary and peduncle tissues placed distal side up on medium supplemented with 2.00 mg l⁻¹ NAA and 10.00 mg l⁻¹ 6-BA. Slices of peduncle tissue were more productive than ovary tissue slices. The productivity of the ovary increased with movement toward the peduncle. No plantlets were obtained from petal, anther, filament, style, leaf or root tissues.

INTRODUCTION

The monocotyledonous plants have traditionally been more difficult tissue culture material than the dicotyledons (2,63,104). Amorphophallus was the first monocotyledon reported to undergo organogenetic differentiation in vitro (57).

Many ornamental bulbous plants belong to a few monocotyledonous families (Amaryllidaceae, Agavaceae, Iridaceae and Liliaceae). Species in these families are often slow to offset naturally and horticultural selections generally produce heterozygous seed. Hippeastrum is no exception to this. The Dutch hybrids at best produce only a moderate number of offsets and a few rarely, if ever, produce any at all (97).

Hippeastrum has probably been selected by large growers for the ability to offset as well as other characteristics. 'Apple Blossom' has been one of the most widely cultivated selections in the world and also one of the freest to offset. Certain of the named cultivars have undergone changes since they were first introduced. It is probable that subtle substitutions have been made that offset more freely or are more easily grown. Infrequent offsetting has been a deterrent to the dissemination of many desirable cultivars. Many species have been difficult to maintain out of their diverse natural environments and are represented by only a very few plants in the possession of a few plant explorers and collectors.

Tissue culture may allow breeders to make commercial use of desirable qualities found in sterile or incompatible hybrids and species.

The increase of these rare plants would be a valuable contribution.

The objective of this investigation was to investigate techniques of in vitro culture which have been used with varying degrees of success in the propagation of other plants, including some bulbous species. Supplements to the culture medium were also investigated for the ability to produce plantlets from different types, location and degree of maturity of Hippeastrum tissues.

The genus Hippeastrum may herein be cited as Amaryllis when used as such by another author. It may also be referred to by this author under the common name amaryllis.

REVIEW OF LITERATURE

Gamborg, et.al. (19) state that the success of any cell tissue culture is governed by two factors: the explant and the culture medium. It might be advantageous to amend this to include culture environment since more factors than the medium exhibit an influence on the explant. The genetic characteristics of the explant are often the limiting factors associated with the ability to obtain proliferation in vitro. One such problem commonly associated with the monocotyledons is the widespread difficulty in obtaining dedifferentiation in mature cells. There are only a few species known to undergo true secondary growth by means of a monocot "cambium" (14). Propagation in vitro may be achieved by direct organogenesis of plantlets from a nonmeristematic explant, induction of growth at a meristematic point or organogenesis from an undifferentiated mass of callus. Clonal reproduction of the main shoot maintains the greatest potential in the leaf axil. However, plants cultured in vitro have a high degree of apical dominance. Thus, the potentially meristematic leaf axils are almost always suppressed. Cytokinins have effectively promoted in vitro "branching" of several monocots. Hussey has demonstrated this on cultivars of Freesia, Gladiolus, Sparaxis and Iris. Branching was profuse on Murashige and Skoog's medium (1962) (MS) containing 6-benzylaminopurine (6-BA) in concentrations of 0.1 ppm. Liliaceae (e.g. Lilium) and Amaryllidaceae (e.g. Narcissus) species proved to be less sensitive to 6-BA. Good branching occurred only at concentrations of 10 ppm and higher (31).

Plantlets of Agave (99), Hyacinthus, Muscari, Ornithogalum (30) and Scilla (30,35) have been regenerated from calli obtained from leaves. Several members of the Liliaceae family Heloniopsis (39,40), Hyacinthus, Muscari, Ornithogalum (30) and Scilla (30,35) have been reported to produce plantlets directly from leaf tissue without first going to callus. Plantlets have been reported to be unobtainable through leaf callus or direct organogenesis of leaf tissues in Hippeastrum, Ipheion, Narcissus, Freesia, Gladiolus, Schizostylis, Sparaxis or Tulipa (30).

Explants of bulb scales from Amaryllidaceae and Liliaceae have been used to increase the rate of propagation for many species. Allium (11), Amaryllis (Hippeastrum) (2), Ipheion (30), Hyacinthus, Muscari (30) and Ornithogalum (30,35) have produced plantlets from bulb scale calli. Sparaxis (30) produced callus that would not differentiate into plantlets. Hussey (30) reports that Hippeastrum bulb-scales will not initiate callus on MS with indole-3-acetic acid (IAA), 1-naphthylacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 0.008 to 8.0 parts 10^{-6} . Bapat and Narayanaswamy (2) obtained Amaryllis bulb-scale callus and ultimately plants on MS + 2,4-D (2 ppm) + kinetin (1 ppm). Vigorous growth of the callus was achieved by the addition of coconut milk (CM) (10% v/v) to the medium. Differentiation of shoot buds was observed in only 10% of the cultures with four to five buds per differentiated culture. The period of culture was not indicated. Two members of Iridaceae [i.e., Freesia (30,58) and Gladiolus (30,85)] have been regenerated from corm callus. Hussey (30) reported that callus from Gladiolus corms developed in vitro but differentiation did not occur.

No cytokinin was added to the medium for Freesia or Gladiolus. Mori et.al. obtained differentiation of Freesia shoots from corm callus on medium containing kinetin (20 mg/l) (58).

Hippeastrum daughter bulbs are naturally initiated only in the axils of the senescing bulb-scales of the outer part of the bulb. The decay of these outer scales releases the daughter bulbs which appear as a ring of "satellite" bulbs around the mother bulb (75). The use of bulb-scales in vitro has proven to be a successful method for the regeneration of bulblets without first going to callus. Luyten (45,46) reported a new method for propagation of Hippeastrum by removal of the leaf-scale from the basal plate and placing it in moist sand. Traub's method (93,94,95) of propagation by means of two bulb-scales joined by a section of basal plate (scale-stem fraction) appears to be superior (103). Crinum (59), Ipheion (30), Narcissus (30,79,81,90), Hyacinthus (30,70,71,91), Lilium (20,21,77,84), Muscari, Ornithogalum (30) and Scilla (30,35) have also been reported to initiate bulblets directly in vitro from leaf-scales. Four members of Iridaceae [i.e., Freesia (30), Gladiolus (30,36,37), Iris (17) and Sparaxis (30)] have also been reported to initiate shoots from nonmeristematic corm pieces. Bulblets of Hyacinthus, Freesia and Narcissus have been longitudinally cut in half and Hippeastrum bulblets have been halved or quartered and cultured in vitro as a means of further increasing the rate of propagation. Each section has the potential to form one bulblet. Hussey (28) reported that the bulblet cuttage cycle for Freesia was 6 to 8 weeks and 12 to 16 weeks for Hippeastrum, Hyacinthus and Narcissus.

Hippeastrum scale sections containing a single leaf-scale and a section of basal plate produced bulblets at the proximal end and roots from the basal plate. Neither roots nor bulblets were regenerated from basal plate sections without the bulb-scale. Bulb-scales obtained from the outer portion of the bulb had a higher rate of regeneration than the juvenile inner scales. No difference in the number of bulblets produced was detected between the thicker part of a single bulb-scale obtained at the base of the assimilatory leaf and the single bulb-scale obtained from the side opposite the base of the assimilatory leaf (101). Hippeastrum leaf bases circumvent the basal plate with the leaf blade positioned directly over the thicker portion of the leaf base. Fujioka (18) found that temperatures of 25°C and 30°C which alternated for 12 hour periods promoted a higher rate of bulblet and root formation in Hippeastrum propagated by cuttage than fixed temperatures of 25°C or 30°C. A temperature of 35°C was inhibitory. Yusof (103) reported that fewer bulblets were formed and decay was a greater problem when the bulb-scales were cultured in total darkness. Bulblet formation was also delayed for 4 additional weeks by the absence of light. Bulblets began to form after 3 weeks under a 12 hour photoperiod provided by cool white and "Gro-Lux" fluorescent bulbs and a light intensity of 300 to 350 ft-c.

Mii, Mori and Iwase (56) reported that when leaf bases were detached from the basal plate of scale-fractions not only the portion immediately adjacent to the basal plate but also portions more distal produced bulblets. The largest number of bulblets was regenerated from bulb-scales in the summer. This was at the time when the bulbs were richest

in total carbohydrates and had a much greater concentration of starches than sugars (78). Likewise, Lilium speciosum has been reported to have a greater regenerative capacity in the summer (77).

Lilium proximal scale sections 1 cm^2 produced larger and a greater number of bulblets than distal sections. Distal sections placed on the abaxial surface produced 4.1 bulblets per explant. Similar sections when placed on the adaxial surface produced only 0.4 bulblets per explant. The difference in regenerative capacity was not nearly as great in the orientation of the proximal sections. The proximal and the distal sections were bisected parallel to their two surfaces and placed cut side down. A total of 12.5 bulblets was produced from the abaxial and adaxial pieces of the proximal and distal sections. An average of three to five bulblets was produced on entire Lilium scales cultured in vitro (20). Morphologically, bulblets of Lilium speciosum were regenerated from mesophyll tissue in the leaf-scale.

Single Hippeastrum bulb-scales produced the largest number of bulblets on the abaxial surface with the adaxial surface up (10). Scale segments containing two thick or two thin scales regenerated bulblets at a higher rate from the position between the scales than from either side regardless of the orientation (101,103). When scale sections were bisected parallel to the two surfaces more than 60% of the adaxial pieces, planted erect or with the cut surface down, produced bulblets at the proximal end. Whole segments cultured in liquid medium on reciprocating and rotary shakers produced bulblets on 17% and 10% of the segments, respectively, on the adaxial surfaces. Yanagawa and Sakanishi suggested

that some regenerative substance which is transmittable through tissue may be more richly concentrated in the abaxial region than the adaxial (101).

Exogenous growth regulators exhibit a synergistic effect on bulb-scales of Lilium. IAA alone shows a greater response for bulblet formation than kinetin. Without IAA, there is little or no response to different levels of kinetin. MS + IAA (10 mg/l) + kinetin (0.1 mg/l) produced more bulblets than with either supplement alone (20). Bapat and Narayanaswamy (2) reported better initiation and growth of Amaryllis explants on 2,4-D and kinetin than on either one singularly. Gibberellic acid (GA_3) and the cytokinin 6-(α,α -dimethylallylamino) purine (2iP) are also reported to have a synergistic relationship. The inhibitory effect of gibberellin on organ formation was not reversed by the anti-gibberellin agents 2,4-dichlorobenzyltributylphosphonium chloride (Phosphon) and N-dimethylaminosuccinamic acid (B-995) or the inhibitor of gibberellin synthesis, 2-chloroethyltrimethylammonium chloride (CCC) but may be countered by high concentrations of cytokinins (13).

The lateral growing points of Asparagus (62,102) have been used for rapid in vitro propagation in the commercial trade. Unfortunately, the apical and lateral growing points of many members of Amaryllidaceae, Iridaceae and Liliaceae are located at or below the soil surface. When this occurs, it becomes very difficult to obtain sterile explants for culture. Hippeastrum (3,34), Narcissus (34,90), Hosta (22), Hyacinthus (34), Lilium (34,43), Fritillaria, Freesia, Gladiolus, Iris, Schizostylis and Sparaxis (34) have all been propagated by induction of

precocious lateral shoots or division of juvenile bulbs or corms. Those species belonging to Amaryllidaceae and Liliaceae required a much higher concentration of cytokinin (6-BA) than members of Iridaceae. Hippeastrum and Fritillaria produced from one to three offsets per plant on MS + 6-BA (2.0 mg l^{-1}). Narcissus and Hyacinthus branched on the same medium with up to 10 branches at 16.0 mg l^{-1} (34). Hosta shoots produced five to six new shoots every 4 to 6 weeks on MS + NAA (1.0 mg/l) + kinetin (2.5 or 5.0 mg/l). 2,4-D produced a poor response (22). Lilium branched on MS + 6-BA (2.0 to 8.0 mg l^{-1}) with one to five laterals produced. Distortion was evident when the 6-BA concentration went over 16.0 mg l^{-1} . Hippeastrum and Fritillaria distortion may become evident at higher concentrations but was not detected at 2.0 mg l^{-1} 6-BA (the highest rate tested). Narcissus and Hyacinthus had little distortion at rates up to 32.0 mg l^{-1} . Sub-cultures could be made in 12 to 18 weeks. Gladiolus, Iris, Schizostylis and Sparaxis were cultured on MS basal medium but went dormant at a period between 6 and 10 weeks. Dormancy was prevented by the addition of 6-BA (0.03 mg l^{-1}) which induced zero to one branches per plant. When the concentration of 6-BA was increased to 0.12 mg l^{-1} one to five branches were induced. Distortion of the shoots and reduction of the root system increased as 6-BA rates went in excess of 0.12 mg l^{-1} . Distorted shoots grew normally when placed on medium low in 6-BA. Freesia did not require 6-BA to prevent dormancy though it did occur after 12 to 14 weeks. This condition was remedied by dividing the corm in half and sub-culturing on the basal medium. Division of the corms

was not successful in reversing dormancy in the other Iridaceous plants. Branching of Freesia was promoted by 6-BA at 0.5 mg l^{-1} or higher. Distortion of the Freesia plantlets was reported in medium supplemented with 6-BA at rates of 8.0 mg l^{-1} or higher (34).

Immature Amaryllis embryos have been cultured by Bell (3) on a modified Hoagland's medium. Mature Iris embryos have been cultured in vitro on MS medium (89). The number of roots was increased by medium supplemented with 3-indolebutyric acid (IBA) (10^{-5} and $5 \times 10^{-5} \text{ M}$) and NAA (5×10^{-7} , 10^{-6} and $5 \times 10^{-6} \text{ M}$). NAA (10^{-6} to 10^{-5} M), IBA (10^{-5} and $5 \times 10^{-5} \text{ M}$) and 2,4-D ($5 \times 10^{-7} \text{ M}$) increased the plant tissue weight. The number of leaves per seedling was increased by kinetin and GA_3 (10^{-5} and $5 \times 10^{-5} \text{ M}$) with the longest roots produced by medium supplements of kinetin (10^{-7} and $5 \times 10^{-7} \text{ M}$) and GA_3 ($5 \times 10^{-6} \text{ M}$) (89).

Lilium (83) plantlets have been obtained from stem-apex callus. Linsmaier and Skoog (1965) medium (LS) (44) was supplemented with 2 mg/l IAA to stimulate callus formation. Callus grown on the agar-based medium produced large numbers of plantlets on LS basal medium when the callus was allowed to accumulate.

Surface-sterilization of sub-surface plant parts has been difficult, particularly when field grown material is used. The use of floral parts, pedicels, receptacles, peduncles and scapes for in vitro culture is of value since these explant sources are relatively free of soil-borne contaminants. Secondly, the use of floral parts does not result in the destruction of the growing point, bulb or corm. These tissues may be obtained while in a young meristematic condition.

Matsubara and Hihara (50) reported that Allium was regenerated from receptacles cut into two or four pieces and cultured on MS medium. The addition of NAA and 6-BA increased the number of plantlets. The juvenile receptacles developed plantlets from the primordia of floral organs. Older receptacles formed plantlets as a result of initiation of adventitious buds. Bulblets were formed in 30 to 90 days.

Hippeastrum peduncle, scape and floral tissues also have potential as good explant materials though results differ and are often contradictory. Bapat and Narayanaswamy (2) with peduncle as propagule have reported that callus was obtained in 8 weeks on MS + 2,4-D (2 ppm) + kinetin (1 ppm). CM (10% v/v) supplements improved the growth of the friable yellow-green callus. Neither NAA nor IAA improved initiation or growth over 2,4-D. Callus had a five-fold increase in 4 weeks. Thirty-five to forty per cent of the cultures regenerated plantlets. Hussey (30) reported "occasional" formation of Hippeastrum plantlets on "stem" pieces after 80 days. The plantlets were generated directly on the tissue (without callus) on MS + NAA (2.0 ppm). Neither IAA nor 2,4-D evoked any response. Pajerski (69) obtained callus and "numerous" bulbs in 10 weeks from scape tissue of Amaryllis 'Red Lion' cultured on LS + NAA (0.3 mg/l). Explants cultured in the dark had 7.7 bulblets per culture while those cultured in the light (16 hours at 150 ft-c and 8 hours dark) had only 6.2 bulbs per culture. No reference was made to the per cent of scape sections producing bulbs or to the number of cultures. Seabrook (80) reports that more plantlets were produced on scape and peduncle disks when the polarity was reversed.

The most productive medium was MS + 2,4-D (1.0 mg/l) + 6-BA (1.0 mg/l). Media which generated shoots but were less productive were MS + NAA (2.4 mg/l) + 6-BA (4.0 mg/l) and MS without auxin or cytokinin supplements. Ninety per cent of the explants of various clones produced 1 to 12 plantlets after 8 weeks. Oyamada (68) reported that no organogenesis occurred on Hippeastrum scape, peduncle or ovary tissues cultured in vitro.

Ipheion (30), Narcissus (79), Freesia (30), Gladiolus (104) and Iris (52,55), Hemerocallis (51,54), Hyacinthus, Muscari, Ornithogalum (30) and Scilla (30,35) have been propagated from peduncle and/or scape calli. Astroloba (49) and Haworthia (41,47,49) have been propagated from flower-axis calli. Schizostylis and Sparaxis (30) have been reported to generate calli though Hussey observed no organogenesis. Hussey failed to obtain callus on peduncle sections from Narcissus (30). This apparent contradiction in results to Seabrook's work with Narcissus scape tissue (79) probably resulted from Seabrook's addition of an exogenous cytokinin to the medium or variations between the types of plant tissue or cultivar response.

Ipheion (30), Narcissus (79,81), Gladiolus (30), Iris (32,33), Schizostylis, Sparaxis (30), Hosta (55), Hyacinthus (30), Lilium (7,9), Muscari, Ornithogalum (30) and Scilla (30,35) generated plantlets directly from peduncle and/or scape tissues. Astroloba (49) and Haworthia (47,49) are reported to generate plantlets from pre-formed buds in flower-axis. Hussey's failure to obtain plantlets from Narcissus by direct organogenesis (30) contradicted Seabrook's (79,81) reported

successes. This variation probably was for the same reasons that Narcissus plantlets were not obtained by Hussey from peduncle callus. Freesia and Tulipa (30) failed to produce plantlets by direct organogenesis from peduncle tissue.

Amaryllis anthers have been reported to form callus but only when associated with exogenous 2,4-D (2). There was no cell division in the microspores but the connective tissue and the anther walls were stimulated to form a callus mass. A 2.33 fold increase by fresh weight took place in 4 weeks of culture. However, only 15 to 20 per cent of the cultures regenerated plantlets. Seabrook (80) reported no response from Hippeastrum anthers even in medium supplemented with 2,4-D (1.0 mg/l). Heimerocallis has been regenerated by means of callus from petals cultured in the dark (8,23,24). Amaryllis perianth showed no growth potential or callus production. The isolated style and filament turned green and elongated but failed to proliferate into a callus mass on MS + CM + 2,4-D (2 ppm) + kinetin (1 ppm) (2). Culture of the ovary tissue (2,30,68) and ovules (2,30) has been reported to be unsuccessful even on MS + 2,4-D + kinetin medium with or without CM supplements (2), but Seabrook reportedly obtained shoots from Hippeastrum ovary explants on MS + NAA (2.0 mg/l) + 6-BA (4.0 mg/l) in only 8 weeks. A few shoots were obtained on MS + NAA (4.0 mg/l) + 6-BA (4.0 mg/l) and MS without exogenous auxin or cytokinin supplements (80).

Ipheion (30), Narcissus (30,79), Haworthia (41,47), Heimerocallis (51), Hosta (53), Hyacinthus, Muscari, Ornithogalum (30) and Scilla (30,35) have been reported to produce plants from ovary or ovule callus.

Ipheion (30), Narcissus (79), Haworthia (47), Hyacinthus, Muscari, Ornithogalum (30) and Scilla (30,35) have been reported to form plant-lets by direct organogenesis from ovaries. Hussey (30) reports that no plants were formed from Narcissus ovary explants. This apparent contradiction with Seabrook (79) may result because Hussey did not supplement the culture medium with a cytokinin. Neither callus production nor organogenesis was observed from ovaries of Tulipa or any members of Iridaceae (Freesia, Gladiolus, Schizostylis, and Sparaxis) (30).

The phenomenon of organogenetic differentiation in plant tissue culture has been found to be the result of interactions between auxins and cytokinins (86). Auxins are credited with the induction of roots and cytokinins are largely responsible for the formation of shoots (1,30).

MATERIALS AND METHODS

Source of experimental plants

Bulbs of Hippeastrum hybridum Hort. cv. 'Apple Blossom' were obtained from a commercial source and cultivated in ground beds in Baton Rouge, Louisiana.

In vitro cultural procedures

All cultures were maintained on Murashige and Skoog's high salt medium (1962) (MS) (Appendix I). The medium was amended with growth regulators and/or organic supplements where indicated. All transfers were made in a laminar-flow hood. Explants were cultured in 25 by 150 mm pyrex tubes closed with metal or plastic closure lids. Explants were cultured under cool-white fluorescent lights radiating 94 micro-einsteins $\text{m}^{-2} \text{sec}^{-1}$ at the rate of 1300 lux. The duration of the photoperiod was 16 hours with 8 hours dark. The temperature was maintained at $21 \pm 1^{\circ}\text{C}$. Unless otherwise stated, all cultures were transferred to fresh medium each 6 weeks.

Bulb cuttage as a source of shoot-apices

Shoot-apices of Amaryllis were obtained from bulb-scales in a water-saturated atmosphere (103) (Plates 1,2,3,4). Mature bulbs of flowering size (20 to 25 cm circumference) were dug and thoroughly washed in water. The roots were trimmed back to the basal plate and the leaves were trimmed back close to the bulb. The bulbs were stripped

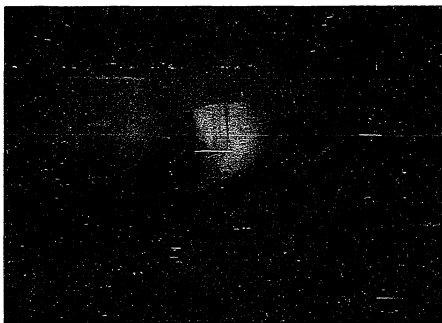


Plate 1. A Hippeastrum bulb showing the stages of cuttage to obtain scale-stem fraction.



Plate 2. A fruit jar with Hippeastrum scale-stem fractions used as a source of shoot-apices.



Plate 3. Bulblets are generally formed on the inner surface of the outer leaf scale and near the basal plate (top) though it may be on the surface of the outer leaf scale (lower right). On some occasions, no bulblets are formed (lower left).

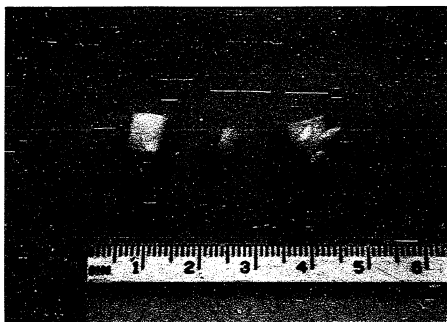


Plate 4. A close up of scale-stem fractions with bulblets (X 1.5)

of the old and desiccated leaf-scales. The bulbs were placed in a benomyl solution (2 g l^{-1}) for 1 hour.

All cutting tools were surface-sterilized in 70 per cent ethanol and flamed. The treated bulbs were vertically sectioned into 10 to 12 radial sections. Each radial section was subsequently chipped into pieces consisting of two bulb scales joined by a portion of basal plate. These bulb pieces have been referred to as "scale-stem fractions" by Traub (97). Each bulb was the source of at least 40 scale-stem fractions.

The scale-stem fractions were dipped in a fresh benomyl solution and placed in quart fruit jars with 1.5 cm of autoclaved distilled water in the bottom (Plate 2). A section of galvanized hardware cloth covered with plastic screen was used to keep the scale-stem fractions out of the water. Each jar containing 25 to 30 fractions was closed with a metal screw cap. A 1 cm hole in the center of each lid was closed with non-absorbent cotton to allow some gas exchange and reduce the chances of fermentation. Thus, the jar's atmosphere was kept at or close to the point of saturation. All jars were stored in the dark at $22 \pm 2^{\circ}\text{C}$.

Culture of excised shoot-apices

Approximately three shoot-apices were formed on each scale-stem fraction. They measured 1.5 to 2.5 mm wide and 2.0 to 3.0 mm tall approximately 3 weeks after the initial cuttage. These shoot-apices consisted of a meristematic dome and two or more leaf primordia. These tissues were excised from the leaf-scale and surface sterilized in a 0.5% aqueous solution of sodium hypochlorite (10% "Clorox") for 10 minutes. The explants were rinsed three times with sterile distilled

water and transferred to MS medium (Appendix I). The medium was supplemented with an auxin [NAA, IAA, 2,4-D or 4-chlorophenoxypropionic acid (4-CPA)] and a cytokinin (kinetin, 6-BA or 2iP) (Table I). Medium was also amended with CCC, NAA and 2iP; GA₃, NAA and 2iP; meso-inositol, thiamine-HCl, pyridoxin-HCl, nicotinic acid and glycine, NAA and kinetin; or CM (Appendix II), NAA and kinetin (Table II).

Propagation by cutting of bulblets

Bulblets 5 to 6 mm in diameter were halved or quartered longitudinally and placed on MS medium supplemented with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and 6-BA (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹). A total of two replications was used for the quartered bulblets and two replications for the halved bulblets. Uncut bulblets of the same size were placed on one replication of medium for the purpose of comparative observation.

Culture of floral tissues

Hippeastrum flowers were excised from mature, naturally cooled bulbs. Flowers were obtained at two periods of development: on December 15 and February 15. Bulbs obtained in December had accumulated 280 hours of chilling (below 7°C). The flower size was determined by the length from the proximal end of the pedicel to the distal end of the unopened petals. The flowers ranged in length from 1.2 to 2.6 cm and averaged 2.3 cm. The bulbs obtained in February had accumulated 870 hours of chilling. Flowers ranged in size from 4.7 to 7.7 cm and averaged 6.0 cm.

Petal, anther, filament and style tissues were excised from surface-sterilized flowers and placed on MS medium. A five by five factorial

Table 1. Auxin cytokinin concentrations used on *Hippeastrum* bulblets cultured in vitro on Murashige and Skoog's high salt medium (1962).

Experiment	Number of treatments ¹	Auxin Conc'n mg l ⁻¹	Cytokinin Conc'n mg l ⁻¹
I	25	NAA ² 0.00, 0.50, 1.00, 2.00, 4.00	kinetin ³ 0.00, 0.50, 1.00, 5.00, 10.00
II	30	NAA 0.00, 1.00, 5.00, 10.00, 15.00	kinetin 0.00, 5.00, 10.00, 15.00, 20.00, 40.00
III	25	NAA 0.00, 0.50, 1.00 2.00, 4.00	2ip ⁴ 0.00, 0.50, 1.00, 5.00, 10.00
IV	25	NAA 0.00, 1.00, 5.00, 10.00, 15.00	2ip 0.00, 5.00, 10.00, 15.00, 20.00
V	25	NAA 0.00, 0.50, 1.00, 2.00, 4.00	6-BA ⁵ 0.00, 0.50, 1.00, 5.00, 10.00
VI	30	NAA 0.00, 1.00, 5.00, 10.00, 15.00	6-BA 0.00, 5.00, 10.00, 15.00, 20.00, 40.00
VII	5	NAA 0.00, 0.50, 1.00, 2.00, 4.00	kinetin 0.33 2ip 0.33 6-BA 0.33 Total 1.00
VIII	25	IAA ⁶ 0.00, 0.50, 1.00, 2.00, 4.00	kinetin 0.00, 0.50, 1.00, 5.00, 10.00
IX	25	IAA 0.00, 0.50, 1.00, 2.00, 4.00	6-BA 0.00, 0.50, 1.00, 5.00, 10.00
X	25	4-CPA ⁷ 0.00, 0.50, 1.00, 2.00, 4.00	6-BA 0.00, 0.50, 1.00, 5.00, 10.00

(continued. . .)

Table 1. (continued. . . .)

Experiment	Number of treatments ¹	Auxin Conc'n mg l ⁻¹	Cytokinin Conc'n mg l ⁻¹
XI	25	2,4-D ⁸ 0.00, 0.50, 1.00, 2.00, 4.00	6-BA 0.00, 0.50, 1.00, 5.00, 10.00
XII	25	2,4-D 0.00, 1.00, 5.00, 10.00, 15.00	6-BA 0.00, 5.00, 10.00, 15.00, 20.00
XIII	25	2,4-D 0.00, 0.50, 1.00, 2.00, 4.00	kinetin 0.00, 0.50, 1.00, 2.00, 4.00

1 The number of treatments is the result of all possible combinations of the concentrations listed. There were four reps per treatment

2 NAA 1-naphthaleneacetic acid

3 Kinetin 6-furfurylaminopurine

4 2ip 6-(γ , γ - dimethylallylamino) purine

5 6-BA 6-benzylaminopurine

6 IAA indole-3-acetic acid

7 4-CPA 4-chlorophenoxypropionic acid

8 2,4-D 2,4-dichlorophenoxyacetic acid

Table 2. Amendments to Murashige and Skoog's high salt medium (1962) for *in vitro* culture of *Hippeastrum* bulblets.

Experiment	Number of treatments ¹	Supplement	Concn mg l ⁻¹	Auxin	Concn mg l ⁻¹	Cytokinin	Concn mg l ⁻¹
XIV	25	CCC ²	0.1	NAA ³	0.00, 0.50, 1.00, 2.00, 4.00	2ip ⁴	0.00, 0.50, 1.00, 5.00, 10.00
XV	25	GA ₃ ⁵	0.1	NAA	0.00, 0.50, 1.00, 2.00, 4.00	2ip	0.00, 0.50, 1.00, 5.00, 10.00
XVI	25	meso-inositol ⁶	200.0	NAA	0.00, 0.50, 1.00, 2.00, 4.00	kinetin	0.00, 0.50, 1.00, 5.00, 10.00
XVII	25	CM ⁸	100,000.0	NAA	0.00, 0.50, 1.00, 2.00, 4.00	kinetin	0.00, 0.50, 1.00, 5.00, 10.00

1 The number of treatments is the result of all possible combinations of the concentrations listed. There were four reps per treatment

2 CCC 2-chloroethyltrimethylammonium chloride

3 NAA 1-naphthaleneacetic acid

4 2ip 6-(γ,δ - dimethylallylamino) purine

5 GA₃ gibberellic acid

6 meso-inositol at two times concentration and thiamine·HCl, pyridoxin·HCl, nicotinic acid and glycine acid at ten times concentration

7 Kinetin 6-furfurylaminopurine

8 CM Coconut milk (Appendix II)

with four replications per treatment was set up to study the effect of auxin (NAA) and cytokinin (kinetin) on these four tissues. The basal medium was amended with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and kinetin (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹) in all possible combinations. Slices of petal tissue measuring 3 mm were taken perpendicular to the axis of the flower and placed distal end down on the medium. The anthers were removed from the filament and both tissues were placed horizontally on the medium. The styles were cut into three segments and also placed horizontally on the medium.

The intact pedicel and ovary tissues from the December flowers were placed on MS basal medium with the distal end down. The pedicel and ovary tissues from the February flowers were separated. Each one was cut horizontally into disks 2 mm thick and placed distal side down on MS medium containing NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and 6-BA (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹).

The pedicel and ovary tissues from December flowers were not transferred to fresh medium. Petal, anther, filament and style tissues were transferred every 6 weeks. The pedicel and ovary slices from the February flowers were transferred each week for 4 weeks then every third week for the duration of the experiment. These cultures were transferred to fresh medium at shorter time intervals than other tissues due to the release of an anthocyanin-like compound from the tissue slices.

Culture of vegetative tissues

The in vitro culture of amaryllis root and leaf tissue was investigated. Root tips of 1.0 cm and leaf disks of 7 mm were surface-sterilized,

excised and placed on MS basal medium supplemented with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and kinetin (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹). Each treatment had four replications. Two replications were on agar-based medium (6.0 g l⁻¹) and two replications were on filter paper bridges in liquid medium.

RESULTS AND DISCUSSION

This work investigated tissue culture methods in their application to the rapid propagation of Hippeastrum. Some methods reported to be used with various degrees of success on Hippeastrum or other species related in some manner to Hippeastrum and other methods of which no reported uses were found were tried.

Nowicki (66) described a method for the freeing of Amaryllis bulbs from virus by use of bulblets from scale-stem fractions. The bulblets did not proliferate upon excision from scale-stem fractions. A system under which the bulblets offset rapidly in vitro would significantly shorten the time needed to build up a large stock of disease-free bulbs. A dependable method of increase would be valuable for use with species and hybrids which are difficult to culture, do not offset rapidly, but are desirable as breeding stock for certain genetic traits.

The basic methods which were used in the investigation have been successful with related families and genera (63,76). Apical growing points, vegetative and floral tissues are responsible for the production of plantlets by direct generation or from masses of calli. This has been reported for species in Agavaceae (99), Amaryllidaceae (2,18,56, 78,80,90), Irideaceae (17,36,52,89,104) and Liliaceae (8,15,22,43,70).

Shoot-apices as an explant source

Shoot-apices of Hippeastrum obtained through the cuttage process were an excellent source of uniform growing points in an active

meristematic condition. Juvenile tissues in vitro have exhibited a strong degree of apical dominance (31). Exogenous sources of growth regulators, particularly the cytokinins, have resulted in axillary branching of plantlets and the induction of shoots from masses of calli (1,13,63,76,100). Hippeastrum, unlike most plants, do not have axillary buds in the leaf axil. Offsets are formed when growing points are induced at the base of the outer leaf-scales. This type of reaction has been reported to be an auxin-cytokinin interaction in other plants (1,65,76,100).

The addition of auxins and cytokinins (Table I) to MS medium on which bulblets were cultured resulted in a failure to offset over a 16 week period. All bulblets grew in a normal manner at a uniform rate regardless of the type of growth regulator or the concentration of the amendment. Concentrations of kinetin (Experiment II) and 6-BA (Experiment VI) were increased to 40.0 mg l^{-1} . Roots failed to form but the bulblets increased in size and normal leaves elongated at a rate equal to other treatments. These results are in contradiction to those obtained by Hussey (34) who reported that bulblets placed on MS medium supplemented with 6-BA (2.0 mg l^{-1}) produced one to three offsets. Steady branching in liquid cultures was obtained for some time when 6-BA was used at 4.0 mg l^{-1} but did not continue beyond four subcultures. Hussey feels that the branching was more adventitious than axillary although it was not always easy to distinguish the difference in monocotyledonous bulbs (38). Hussey (31) also states that species which belong to Liliaceae (e.g. Lilium) and Amaryllidaceae (e.g. Narcissus) are much less sensitive to 6-BA than other monocotyledonous families.

Good branching of Lilium and Narcissus occurred only at concentrations of 10 ppm or more.

The erratic effects and unpredictability of cytokinin in culture (38) may have been the biggest factor in the variation between results from this work and Hussey's. Variation between cultivars has been a factor affecting other families and may have been of considerable influence on Hippeastrum. The effect of climate and cultural conditions on the parent material must be considered. The same cultivar maintained under different cultural conditions may display different natural reproductive characteristics. Thus, it is possible that certain growth regulators were or were not present in the explant source used by Hussey.

Yanagawa and Sakanishi (101) have suggested that a transmittable regenerative substance exists which was responsible for the formation of Hippeastrum bulblets on the scale-stem fractions. This undefined endogenous growth regulator exhibited a cytokinin-like effect on the tissues though it was probably not a cytokinin. If the offsetting had been a cytokinin response, the bulblets should have responded to 6-BA, kinetin or 2iP within the concentrations tested.

The possibility exists that the bulblets were retarded from offsetting by high concentrations of endogenous gibberellins acting as an inhibitor of organ formation. The inhibitor of gibberellin syntheses, CCC (0.1 mg l^{-1}) failed to induce offsetting even when associated with NAA ($0.00, 0.50, 1.00, 2.00$ or 4.00 mg l^{-1}) and 2iP ($0.00, 0.50, 1.00, 5.00$ or 10.00 mg l^{-1}) (Experiment XIV). The addition of GA_3 (0.1 mg l^{-1}) with NAA ($0.00, 0.50, 1.00, 2.00$ or 4.00 mg l^{-1}) and 2iP ($0.00, 0.50,$

1.00, 5.00 or 10.00 mg l⁻¹) (Experiment XV) resulted in the same rate and type of growth as the control and medium supplemented with CCC, NAA and 2iP. Engelke et.al. (13) stated that the inhibitor of gibberellin synthesis, CCC, was not effective in counteracting the inhibitory effect of gibberellin on organ formation in tobacco callus in vitro. However, high concentrations (25.0 µM) of cytokinins (kinetin or 2iP) counteracted this effect. Thus, it was concluded that the failure of Hippeastrum bulblets to differentiate adventitious or axillary offsets was not the result of the inhibitory effect of gibberellin.

Increased concentrations of vitamins

Medium amended with higher rates of vitamins has been reported to show increased vigor in the growth of some tobacco calli cultures (44). MS medium was amended with a two-fold increase in meso-inositol (200.0 mg l⁻¹) and a 10-fold increase in thiamine·HCl (1.0 mg l⁻¹), pyridoxin·HCl (5.0 mg l⁻¹), nicotinic acid (5.0 mg l⁻¹) and glycine (20.0 mg l⁻¹). Bulblets were placed on the amended MS medium and cultured as previously described. Neither the growth of the bulblets nor the tendency to offset was stimulated or retarded by the higher than normal vitamin levels.

Propagation by cutting of bulblets

Bulblets 5 to 8 mm in diameter were halved or quartered longitudinally and placed on MS medium supplemented with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and 6-BA (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹). A total of two replications was used for the quartered bulblets and

two replications for those halved. Uncut bulblets of the same size were placed on one replication of medium for the purpose of comparative observation.

Scale-stem fractions were allowed to remain in the water-saturated atmosphere for 6 weeks in order that bulblets 5 to 6 mm could be obtained. It was necessary that bulblets be allowed to reach this size range so that each bulblet fraction had a section of basal plate attaching the minute leaf scales. The surface-sterilized bulblets were halved or quartered and placed in an upright position. In spite of careful treatment, some bulbs became horizontal.

All bulblet fractions in spite of position or treatment formed one new bulblet each. The original size of the mother bulblet was reached between the 10th and 12th week. No variations in culture time or size were detected between the different levels of NAA and 6-BA. The control group of 25 bulblets had obtained a size range of 8 to 10 mm in diameter but had not divided.

A 20 to 25 cm bulb will yield at least 40 scale-stem fractions. Each scale-stem fraction will produce an average of 2.5 bulblets at the end of 6 weeks in the water-saturated atmosphere. The quartering and subculturing process may be carried out each 10 to 12 weeks. If an average figure of 11 weeks is to be used, a theoretical increase in excess of 25,000 bulblets may be obtained one year after the original mother bulb is cut. Thus, a 250 fold increase will be obtained over the standard scale-stem fraction process. A commercial grower should realize an increase of about 10,000 fold over natural offsetting.

Roots were formed from the basal plate without special treatment within 2 to 4 weeks after the 5 to 6 mm stage was reached. However, roots formed about 1 to 2 weeks earlier at the upper two concentrations of NAA, regardless of the 6-BA concentration. Bulblets in the 5 to 6 mm range were transplanted with and without roots with equal success. A peat-lite mix in 57 mm plastic pots proved to be satisfactory as an in vivo culture medium. The bulbs were maintained at or near 100 per cent relative humidity. An 18 hour photoperiod of 2,500 lux and 360 microeinsteins $\text{m}^{-2} \text{sec}^{-1}$ was provided each 24 hours by half and half "Gro-Lux" and cool-white fluorescent bulbs. The temperature was maintained at $30 \pm 2^{\circ}\text{C}$.

Culture of floral tissues

The use of floral tissues offers four major advantages for the rapid in vitro propagation of Hippeastrum. First, it does not require the destruction of the bulb. Second, explant material may be obtained at the time of flowering, which ensures that only true-to-type material is being propagated. Third, this enables a breeder to propagate unique seedlings as soon as they are deemed desirable without destruction of the bulb. Finally, explants obtained from the above-ground parts of bulbous plants are relatively free of soil-borne contaminants (8,24,41, 52,54,104).

Petal, anther, filament and style tissues were excised from surface-sterilized flowers and placed on MS medium supplemented with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l^{-1}) and kinetin (0.00, 0.50, 1.00, 5.00 or 10.00 mg l^{-1}). Squares of petal tissue (8 by 8 mm) rolled up in a manner

which resulted in the loss of contact with the medium. This resulted in rapid desiccation and senescing of the tissue. Slices of petal tissue measuring 3 mm thick were taken perpendicular to the axis of the flower and placed distal end down on the medium. These tissues remained in contact with the medium. They increased in size by about four fold over a 4 week period. The increase in size was attributed to cell enlargement and not to division after microscopic observation. The tissues remained alive for more than 9 months but did not form callus or plantlets. No variation was observed between the age of the tissue or the location on the flower. Anther, filament and style tissues did not enlarge like the petal tissue. The anthers dehisced along the suture after 10 days of culture but no pollen was shed. Specks of yellow-green to cream colored callus about 0.5 to 1.0 mm were produced at the ends of the filament and style tissues on medium which contained auxin. Callus was not formed at the zero auxin level and did not appear to be influenced by the level of cytokinin. The callus could not be increased even on medium supplemented by 10% (v/v) CM. No variation was observed between the two ages of floral tissue tested (December and February). Bapat and Narayanaswamy (2) reported that Amaryllis callus could be grown on MS medium supplemented with 10% (v/v) CM. One possible reason for this discrepancy was that NAA was the auxin used to initiate callus in this experiment instead of 2,4-D, used by Bapat and Narayanaswamy.

Explants consisting of the entire peduncle and ovary of seven December flowers were placed distal end down on MS medium supplemented with NAA (1.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}). The medium became

reddish-brown to almost black in color after a period of 8 weeks in culture. The ovary portion began to swell at the 28th week and continued to increase in size until the 32nd week when the ovaries dehiscid along the sutures. A total of 52 bulblets was obtained from five of the seven explants (Plate 5). The remaining two explants produced no plantlets. The bulblets had formed from the inside of the ovary wall and possibly on the placenta. The explant had degenerated by this time to a leathery mass and showed no indication of further differentiation. However, the bulblets only rarely rooted until they were removed from this residue (Plate 6). The level of auxin and cytokinin did not appear to affect the formation of roots which formed equally well at all concentrations of NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and kinetin (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹).

Slices of peduncle and ovary tissues 2 mm thick were taken perpendicular to the axis of the flower. The slices were placed distal end down on MS medium amended with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and 6-BA (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹). The explants had begun to form a white non-firable callus on the proximal cut of the peduncle and the ovary slices by the fifth day of culture. By the ninth day, the slices had formed a dense crust of callus (Plates 7,8, 9,10,11). The ovules in the ovaries enlarged to 2 mm in the second week but did not develop into callus or plantlets. They remained white and enlarged until the 12th week when they began to turn brown and degenerate. Growing points were clearly visible on the peduncles by the 24th week.

The release of an anthocyanin-like material from the peduncle and ovary explants appears to be correlated to the condition of the tissue



Plate 5. Bulblets formed on entire peduncle and ovary tissue of a December flower 36 weeks after being excised from the flower MS + NAA (1.0 mg l^{-1}) + kinetin (1.0 mg l^{-1}).



Plate 6. Bulblets inhibited from rooting by the peduncle residue MS + NAA (1.00 mg l^{-1}) + kinetin (1.00 mg l^{-1}).

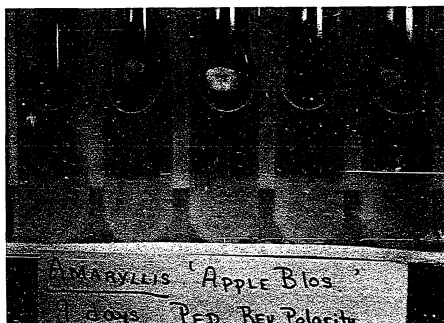


Plate 7. *Hippeastrum* (*Amaryllis*) peduncle tissue after 9 days on MS medium supplemented with kinetin (0.00 mg l^{-1}) and NAA ($0.00, 0.50, 1.00, 2.00$ and 4.00 mg l^{-1}) (left to right).

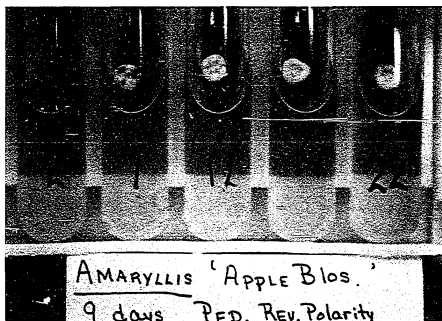


Plate 8. *Hippeastrum* (*Amaryllis*) peduncle tissue after 9 days on MS medium supplemented with kinetin (0.50 mg l^{-1}) and NAA ($0.00, 0.50, 1.00, 2.00$ and 4.00 mg l^{-1}) (left to right).

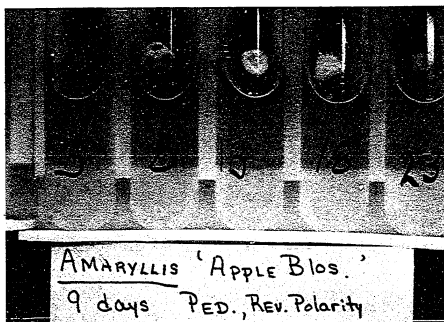


Plate 9. *Hippeastrum* (*Amaryllis*) peduncle tissue after 9 days on MS medium supplemented with kinetin (1.00 mg l^{-1}) and NAA ($0.00, 0.50, 1.00, 2.00$ and 4.00 mg l^{-1}) (left to right).

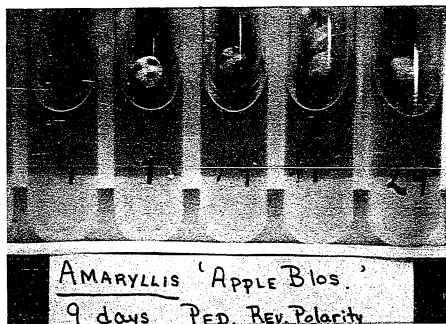


Plate 10. *Hippeastrum* (*Amaryllis*) peduncle tissue after 9 days on MS medium supplemented with kinetin (5.00 mg l^{-1}) and NAA ($0.00, 0.50, 1.00, 2.00$ and 4.00 mg l^{-1}) (left to right).

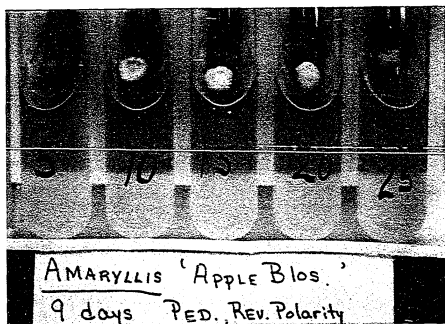


Plate 11. *Hippeastrum* (*Amaryllis*) peduncle tissue after 9 days on MS medium supplemented with kinetin (10.00 mg l^{-1}) and NAA ($0.00, 0.50, 1.00, 2.00$ and 4.00 mg l^{-1}) (left to right).

and apparently was not toxic to the explant. Those tissues on medium containing the zero level of NAA did not discolor the medium after the first transfer. Likewise, after the third weekly transfer, those tissues on medium with 0.5 mg l^{-1} NAA ceased to release the exudate. These tissues began to visibly degenerate between 1 and 2 weeks after the exudation stopped. The greatest amount of medium discoloration was seen with 6-BA at 10.0 mg l^{-1} and NAA above 0.5 mg l^{-1} . This roughly corresponds to the conditions under which the greatest number of plants were produced. When young actively growing bulblets were transferred to old medium discolored by the anthocyanin-like material they grew as rapidly as bulblets on fresh medium.

The number of plants and roots produced by the ovary tissue increased with movement from the distal to the proximal sections. Shoots formed when NAA and 6-BA concentrations were respectively 1.0 and 10.00, 2.0 and 5.00, and 2.0 and 10.00 mg l^{-1} . The later formulation was the most effective. The plantlets produced on the peduncle like those on the ovary were formed from the inner tissues at the proximal end. The NAA and 6-BA concentrations were the same for plantlet production. However, the peduncle tissues were more prolific than the ovary tissues (Plates 12,13). This investigation of 'Apple Blossom' has resulted in plantlets from peduncle and ovary tissues of both the February flowers showing a blush of color as well as the very juvenile December tissues. This is opposite to Meyer's (54) results with Hemerocallis where plantlets were often produced only in the peduncle a few millimeters below the ovary.



Plate 12. Bulblets formed on ovary tissue 28 weeks after culture on MS + NAA (1.00 mg l^{-1}) + 6-BA (5.00 mg l^{-1}).

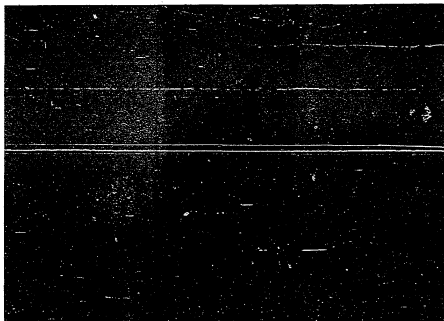


Plate 13. Bulblets formed on peduncle tissue 28 weeks after culture on MS + NAA (2.00 mg l^{-1}) + 6-BA (10.00 mg l^{-1}).

Culture of vegetative tissues

Root tips (1.0 cm) cultured in vitro on MS medium (using both filter paper bridges and agar base) supplemented with NAA (0.00, 0.50, 1.00, 2.00 or 4.00 mg l⁻¹) and kinetin (0.00, 0.50, 1.00, 5.00 or 10.00 mg l⁻¹) did not form callus, plantlets or enlarge (Plate 14). The 3 mm directly behind the root cap did become dark green. The explants remained alive for a period of 6 months before senescing.

Leaf disks (7 mm) were obtained from leaf blades which had extended to 15 cm above the basal plate. The disks were taken from the 6 to 13 cm zone and placed adaxial side up on MS medium as described for the root tips. The disks turned a reddish-brown by the end of the first week of culture. After 4 weeks of culture, the explants had almost tripled their original thickness. The 4 mm thick disks were leathery to woody in nature (Plate 15). The explants degenerated until the fifth month when they became senescent. No callus or explants were produced by any treatment. No variation was noted between filter paper bridges and agar based medium in either the root tip or the leaf disk trials.



Plate 14. Hippeastrum root tip cultured on filter paper bridge.

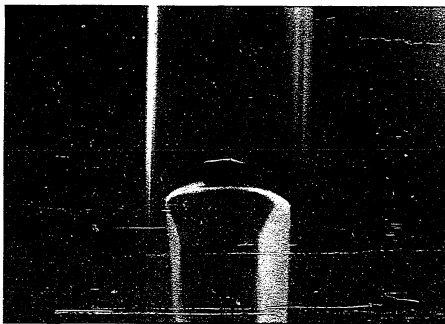


Plate 15. Hippeastrum leaf disk cultured on filter paper bridge.

SUMMARY AND CONCLUSIONS

Hippeastrum hybridum Hort. cv. 'Apple Blossom' may be rapidly propagated from bulblets obtained from scale-stem fractions. The rate of propagation may be further increased by longitudinal quartering of the bulblets. A 250 fold increase may be realized over the scale-stem fraction process. Bulblets obtained from scale-stem fractions could not be induced to divide in vitro by auxin and/or cytokinin supplements to the medium.

Plantlets were obtained from 2 mm slices of ovary and peduncle tissues and placed distal side up on Murashige and Skoog's high salt medium supplemented with 2.00 mg l⁻¹ NAA and 10.00 mg l⁻¹ 6-BA. Slices of peduncle tissue were more productive than ovary tissue slices. The productivity of the ovary increased with movement toward the peduncle. No plantlets were obtained from petal, anther, filament, style, leaf or root tissues.

The major problem of plant tissue culture research has been the variability of results between researchers. In vitro propagation of Hippeastrum has been no exception. The inherent genetic variation between clones, the grouping of similar lines into clones and the variation between techniques may have been factors leading to different results. Relatively little has been learned of the effect of the cultural environment on the explant and its response in vitro. Statistical chance should have little effect with the rigidly controlled

environment of a culture room but apparently does.

In vitro culture of ornamental plants has a bright future as the public increases its demand for disease free named cultivars. To meet these needs, it will be necessary to resort to tissue culture for the techniques of rapid propagation. In vitro work on Hippeastrum as in this investigation shows promise as a method to reduce time involved in developing new cultivars and as a tool for the mass production of sterile species and hybrids slow to offset.

APPENDIX I

Murashige and Skoog's (1962) high salt nutrient medium for the formation and growth of plantlets from explants of Hippeastrum (60).

<u>Ingredients</u>	<u>mg l⁻¹ medium</u>
<u>Inorganic constituents</u>	
NH ₄ NO ₃	1,650.0
KNO ₃	1,900.0
CaCl ₂ ·2H ₂ O	440.0
MgSO ₄ ·7H ₂ O	370.0
KH ₂ PO ₄	170.0
NaH ₂ PO ₄ ·H ₂ O	170.0
Na ₂ EDTA	37.3
FeSO ₄ ·7H ₂ O	27.8
H ₃ BO ₃	6.2
MnSO ₄ ·4H ₂ O	22.3
ZnSO ₄ ·4H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ ·2H ₂ O	0.25
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025
<u>Organic constituents</u>	
Thiamine·HCl	1.0
Pyridoxin·HCl	5.0
Nicotinic Acid	5.0
Meso-Inositol	100.0
Adenine sulfate-dihydrate	40.0
Sucrose	25,000.0
Difco Bacto agar	6,000.0

APPENDIX II

Preparation of coconut milk (coconut water)
for use as medium supplement in plant tissue
cultures (98).

The procedure used in the preparation of coconut milk as a liquid
supplement for tissue culture medium is as follows:

1. Remove the liquid endosperm from the coconuts
2. Heat to 80°C to fix it
3. Cool and store at -15°C
4. Add to the medium at the rate of 10% (v/v)
5. Autoclave the medium for 15 minutes at 1.1 kg cm⁻²
pressure and 121°C

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VITA

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EXAMINATION AND THESIS REPORT

Candidate: William Maynard Fountain, III

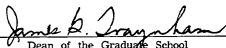
Major Field: Horticulture

Title of Thesis: Investigations of in vitro Culture of Hippeastrum as a Method of Rapid Propagation

Approved:

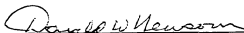


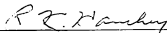
Major Professor and Chairman

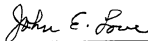



Dean of the Graduate School

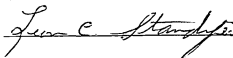
EXAMINING COMMITTEE:











Date of Examination:

March 16, 1979